Dictyostelium Myosin Heavy Chain Kinase A Regulates Myosin Localization during Growth and Development

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Abstract. Phosphorylation of the *Dictyostelium* myosin II heavy chain (MHC) has a key role in regulating myosin localization in vivo and drives filament disassembly in vitro. Previous molecular analysis of the *Dictyostelium* myosin II heavy chain kinase (MHCK A) gene has demonstrated that the catalytic domain of this enzyme is extremely novel, showing no significant similarity to the known classes of protein kinases (Futey, L. M., Q. G. Medley, G. P. Côté, and T. T. Egelhoff. 1995. *J. Biol. Chem.* 270:523–529). To address the physiological roles of this enzyme, we have analyzed the cellular consequences of MHCK A gene disruption (*mhck A*$^-$ cells) and MHCK A overexpression (MHCK A^{++} cells). The *mhck A*$^-$ cells are viable and competent for tested myosin-based contractile events, but display partial defects in myosin localization. Both growth phase and developed *mhck A*$^-$ cells show substantially reduced MHC kinase activity in crude lysates, as well as significant overassembly of myosin into the Triton-resistant cytoskeletal fractions. MHCK A^{++} cells display elevated levels of MHC kinase activity in crude extracts, and show reduced assembly of myosin into Triton-resistant cytoskeletal fractions. MHCK A^{++} cells show reduced growth rates in suspension, becoming large and multinucleated, and arrest at the mound stage during development. These results demonstrate that MHCK A functions in vivo as a protein kinase with physiological roles in regulating myosin II localization and assembly in *Dictyostelium* cells during both growth and developmental stages.

NONMUSCLE myosin II is a major component of the cytoskeleton in eukaryotic cells, and is involved or implicated in a wide range of contractile events (34). In *Dictyostelium discoideum*, genetic and cellular studies have demonstrated essential roles for myosin II in cytokinesis, multicellular development, and receptor capping (7, 12, 27). These studies have also demonstrated central roles for myosin II in cell locomotion and in maintenance of cortical tension (17, 27, 38). Although the roles of myosin II in nonmuscle cells are generally becoming clear, the mechanisms regulating localization and activity of myosin II in these settings are not well understood. Myosin light chain (MLC)[1] phosphorylation has a clear role in regulating mammalian smooth and nonmuscle myosin II motor function, and is also thought to participate in regulation of filament assembly in nonmuscle cells (36). Although phosphorylation of the myosin II heavy chain (MHC) has also been observed in several types of mammalian nonmuscle cells in a variety of settings (4, 18, 23, 26), the definitive biochemical consequences of MHC phosphorylation in the mammalian systems are not known.

In *Dictyostelium*, MHC phosphorylation plays a key role in regulating filament assembly both in vitro and in vivo. Two distinct MHC kinases (MHCKs) have been purified to homogeneity from *Dictyostelium*. A 84-kD MHCK purified by Ravid and Spudich (28) is expressed only during development. A 130-kD MHCK (MHCK A) purified by Côté and Bukiejko (5) is expressed during both growth and developmental stages (Medley, Q. G., S. F. Lee, G. A. Cates, and G. P. Côté. 1993. *J. Cell Biol.* 115:29*a*). Both the 130-kD MHCK A and 84-kD MHCK phosphorylate myosin on threonine residues and can drive myosin filament disassembly in vitro (6, 28). The 130-kD MHCK A phosphorylates threonine residues 1823, 1833, and 2029 in vitro (22, 37). The 84-kD MHCK phosphorylates sites within the carboxyl-terminal portion of the myosin molecule (*7a*, *26a*), but its exact target sites have not been mapped. In addition to these two threonine-specific MHCKs, evidence exists for other MHC kinase activities with specificity towards threonine and serine (20, 25). However, it is not known whether these other in vitro activities have physiological roles in regulating myosin function.

The in vivo importance of the mapped MHCK A target sites on the MHC has been demonstrated in studies in which the target sites were converted to either alanine (3X ALA myosin) or to aspartic acid (3X ASP myosin) resi-

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1. *Abbreviations used in this paper*: IP, immunoprecipitation buffer; MLC, myosin light chain; MHC, myosin II heavy chain; MHCK, MHC kinases; MHCK A, 130-kD MHCK.

dues (11). These substitutions create either a nonphosphorylatable MHC, or pseudophosphorylated MHC, respectively. In vitro the 3X ALA myosin assembles into bipolar filaments as efficiently as wild-type myosin while the 3X ASP myosin is unable to assemble at physiological salt conditions. In vivo 3X ALA myosin dramatically overassembles into the cytoskeleton, while 3X ASP myosin is underassembled relative to wild-type cells. Cells expressing the 3X ALA myosin are competent for myosin dependent contractile events such as cytokinesis and development, although partial defects are apparent. In confocal microscopy studies, cells expressing the 3X ALA MHC display a dramatic over localization of myosin to the cortical regions of the cells (14). In contrast, cells expressing the 3X ASP MHC are phenotypically identical to MHC null cells (*MHC*$^-$), indicating that pseudophosphorylated MHC cannot participate in cellular force production. These studies indicate that the mapped MHCK A target sites play a key role in regulating myosin II assembly into the cytoskeleton and that this assembly is required for myosin function in vivo.

At the primary sequence level the 84-kD and 130-kD MHCKs are unrelated. The 84-kD enzyme displays significant similarity to members of the Protein Kinase C (PKC) family, particularly within the catalytic domain (29). In contrast, the primary sequence of the 130-kD MHCK A displays no significant similarity to any members of the conventional classes of eukaryotic protein kinases. The protein consists of an amino-terminal domain predicted to have an α-helical coiled-coil structure, a central domain believed to contain the catalytic functions, and a carboxyl-terminal domain which bears significant similarity to the β-subunit of heterotrimeric G proteins (13). In view of the novelty of this enzyme, the MHCK A gene was expressed in *E. coli,* and studies confirmed that the recombinant protein was able to autophosphorylate and to phosphorylate the *Dictyostelium* MHC (13).

A major unresolved question concerning myosin regulation in *Dictyostelium* is the physiological role of the multiple unrelated MHCKs. The studies reported here were designed to elucidate the role that the novel 130-kD MHCK A has in vivo by analyzing MHCK A null cell lines and cell lines overexpressing the cloned MHCK A gene. Data obtained with MHCK A null cell lines and cell lines overexpressing the cloned MHCK A gene demonstrate that this enzyme does exhibit MHC kinase activity in vivo, and that it influences myosin localization and assembly during both growth and development.

Materials and Methods

Dictyostelium Growth and Development

Dictyostelium discoideum strain JH10 (15) was employed as the parental cell type for the studies described here. Cells were cultured axenically on plastic petri dishes in HL5-medium (35) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml thymidine. For suspension growth analysis cells were transferred from petri dishes to small Erlenmeyer flasks for ~24 h to allow adaptation to suspension growth, and then adjusted to a low cell density. Cultures were rotated at 200 rpm at 21°C and cells were counted daily. Each cell line was inoculated in triplicate and counted independently. Doubling times were determined for cultures during log phase growth, and values from each triplicate culture averaged. For developmental studies cells were harvested in log phase growth and plated on filter membranes as described (35). The cell line HS1 (31) was used as an *MHC*$^-$ control for some experiments.

Plasmid Constructs and Transformations

All DNA manipulations were performed using standard laboratory methods (32). Brief description of plasmid constructions are given below. Full details of all constructs are available upon request. To construct an MHCK gene replacement cell line, a gene replacement vector was made as follows. A 0.9-kb ClaI–AccI fragment containing the 5′ flanking region of *mhck A* gene, and a 1.1-kb ClaI–EcoRI fragment containing the 3′ portion of the gene were isolated from size selected plasmid libraries of *Dictyostelium* DNA using the *mhck A* cDNA as a probe. The gene replacement vector, pDF15, was generated by ligating the 0.9-kb ClaI–AccI fragment and 1.1-kb ClaI–EcoRI fragment into the vector pTZ18 (Pharmacia Biotech) with the *Dictyostelium Thy1* gene (9) between the genomic fragments. The *Thy1* gene was isolated from the plasmid pThy-Short (a gift from K. Ng and G. Nuckolls, Stanford University). The plasmid pThy-Short contains the *Thy1* marker isolated by Dynes and Firtel (9), except that 1.1 kb of genomic DNA upstream of the *Thy1* promoter has been removed by Exonuclease III and S1 nuclease digestion as follows. The 3.2-kb *Thy1* marker from pGEM26 was cloned as an SalI fragment into the XhoI site of pGEM7 (Promega, Madison, WI). This plasmid was digested with EcoRI and SacI, which both cut the polylinker on the 5′ end of the *Thy1* fragment. Several progressive deletions of the *Thy1* fragment were created using the Erase-a-Base system (Promega). pThy-Short was the smallest fragment produced in this approach that complimented the *Thy*$^-$ defect when transformed into the *Dictyostelium* cell line JH10.

Plasmid pDF15 was linearized at polylinker restriction sites immediately adjacent to the 5′ and 3′ segments of homology to promote recombination at the *mhck A* locus. The thymidine-auxotrophic cell line JH10 was electroporated with 5 g of restricted pDF15 and transformants were selected in HL-5 medium lacking thymidine. Colonies were picked around the seventh day and transferred to 12-well microtiter plates. The isolated colonies were screened for the absence of MHCK A by Western blotting using an anti-MHCK A monoclonal antibody (13). Of fourteen independent isolates originally screened, twelve showed no reactivity with the anti-MHCK A antiserum. Four of these cell lines were analyzed by Southern blotting and all appeared to be gene replacement events generated via double recombination at the 5′ and 3′ homology segments. JH10 cells transfected with pThy-Short (referred to as JH10 *thy*$^+$ cells) were used as a control for cell biological studies of *mhck A*$^-$ cells.

Southern Blot Analysis

Genomic Southern blot analysis of the *mhck A* locus was performed on parental (JH10) and four independent transformed cell lines that had tested negative for MHCK A expression by Western blot analysis. DNA was isolated and purified by CsCl density gradients from parental and MHCK A null cell lines. After digestion of DNA with XbaI, 0.6 μg DNA was loaded onto a 0.8% agarose gel, electrophoresed, and transferred to GeneScreen Plus membrane (New England Nuclear Research Products, Boston, MA). The filters were hybridized with ^{32}P-labeled cDNA probes following GeneScreen Plus protocols. Probe A is a 1.1-kb ClaI–BclI fragment containing 0.9 kb of *mhck A* 5′ flanking region and 0.2 kb of *mhck A* coding sequence. Probe B corresponds to a 1.7-kb KpnI–KpnI restriction fragment of the *mhck A* cDNA.

MHCK A Overexpressing Cells

The *Dictyostelium mhck A* cDNA was fused to the *Dictyostelium* actin 15 promoter, so that codon 8 of the actin 15 gene is fused to codon 7 of *mhck A*. This modified *mhck A* gene was then cloned into the expression vector pLittle (constructed by Dr. T. Q. P. Uyeda, NAIR, Japan, unpublished) to generate pLMHCK. pLittle is a derivative of pBIG (31) which contains part of the native *Dictyostelium* extrachromosomal plasmid Ddp 1, a G418 resistance cartridge, and *E. coli* replication functions. The plasmid pLittle differs from pBIG in that a 1.5-kb KpnI fragment in the Ddp1 portion of pBIG was removed. The plasmid pLMHCK was introduced into the *mhck A*$^-$ cell line by electroporation, grown overnight in HL5, and then transformants were selected in the presence of 8 g/ml G418. Colonies were picked after ~5 d, transferred to microtiter plates for clonal growth, and then screened for the presence of MHCK A by Western blot analysis. In early studies it was demonstrated that the actin 15 promoter expression level increases significantly during development (19). In more recent studies using this promoter to drive myosin expression from extrachromo-

somal plasmids (10, 11, 21), significant expression has always been observed during growth phase as well.

Generation of Polyclonal Antiserum

A portion of the MHCK A cDNA corresponding to amino acids 7-656 was expressed in *E. coli* using the expression vector pET21d (Novagen, Madison, WI). The resultant fusion protein contains a $(His)_6$ carboxyl-terminal tag which allows purification using metal ion affinity chromatography methods as described by the pET21 vector supplier. Approximately 200–300 g of affinity-purified protein was injected into a New Zealand white rabbit at 4-wk intervals, with serum collected 12–14 d later. Cleared serum was used at 1:1,000 dilution for Western blots.

Polyclonal antiserum against *Dictyostelium* myosin was made by injecting rabbits with purified myosin using a similar schedule to that described above. For Western blot analysis the anti-myosin antiserum was used at a 1:1,000 dilution, followed by alkaline phosphatase conjugated secondary antibody, and development with BCIP (5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt) and NBT (*p*-nitro blue tetrazolium chloride) (16).

Western Blot Analysis

Cells were harvested from plates, washed with 15 mM Tris, pH 7.5, and resuspended in buffer containing 50 mM Tris, pH 8.0, 20 mM NaPPi, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 1 g/ml leupeptin, 1 g/ml pepstatin, and 10 g/ml PMSF. An equal volume of 2× Sample buffer (50 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 50 l/ml 2-mercaptoethanol, and 0.2 g/ml bromophenol blue) was then added and samples were then immediately boiled for 5 min and subjected to SDS-PAGE. Samples were either stained with Coomassie blue or transferred to nitrocellulose and probed with MHCK A monoclonal or polyclonal antibodies. Signal was detected using either an alkaline phosphatase-linked goat anti-rabbit antibody or ^{125}I-goat anti-mouse antibody.

Myosin Phosphorylation and Immunoprecipitation

Myosin was phosphorylated in cell lysates as previously described by Berlot et al. (2), with modifications described below. Cells were washed with 15 mM Tris, pH 7.5. 150 μl of cells (3×10^6) in 15 mM Tris were added to 150 μl of a reaction mixture containing 0.2% Triton X-100, 4 mM $MgCl_2$, 4 mM $MnCl_2$, 7.5 mM Tris, 10 μM ATP, and 100 Ci/mmol [γ-^{32}P]ATP and incubated on ice for 5 min. Reactions were stopped by adding an equal volume of either ice-cold 5% TCA or 2 × immunoprecipitation buffer (IP) containing 150 mM NaCl, 1% Triton X-100, 15 mM Tris-HCl, pH 7.5, 2 mM NaN_3, 5 mM EDTA.

Pansorbin (Calbiochem) (50 l) and anti-myosin polyclonal antiserum (10 l) were mixed with IP buffer (100 l), rotated at 4°C for 4–18 h, and washed three times with IP buffer. Myosin was immunoprecipitated using denaturing conditions. An equal volume of 100 mM Tris, pH 8.0, 10 mM EGTA, 10 mM EDTA, 2% SDS, 4 mM ATP, 100 mM NaFl, 50 mM $NaPP_i$, 10 μM okadaic Acid, 20 μg/ml pepstatin, 50 μg/ml L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl (TLCK), and 10 μg/ml PMSF) was added to the lysates and samples were immediately boiled for 10 min. Pansorbin (4 μl) was added and the lysates were cleared by centrifugation at top speed in a microfuge for 5 min. The supernatant was transferred to a fresh tube containing 1 ml of IP buffer containing the preadsorbed Pansorbin-myosin polyclonal antibody complex described above and mixed overnight with end-over-end rotation at 4°C. The immunoprecipitated myosin was washed as described in (30) with minor modifications. Briefly, the Pansorbin pellet was washed two times with IP buffer containing 0.2% SDS, washed two times with 2 M urea, 200 mM NaCl, 1% Triton X-100, 100 mM Tris-HCl, pH 7.5, 2 mM NaN_3, 5 mM EDTA, once with 500 mM NaCl, 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 2 mM NaN_3, 5 mM EDTA, and once with 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM NaN_3, 5 mM EDTA. The final complex was then boiled in SDS sample buffer, spun to remove the Pansorbin and soluble material was then subjected to SDS-PAGE.

Phosphoaminoacid Analysis

After SDS-PAGE, myosin was transferred electrophoretically to Immobilon P membrane (Millipore, Bedford, MA) and stained with Ponceau to visualize protein bands. The myosin band was excised, wetted in methanol and rinsed with 1 ml water three times to remove any residual transfer buffer. Phosphoaminoacid Analysis (PPA) was performed according to Boyle et al. (3). The myosin was hydrolyzed with 5.7 N HCl and incubated for 4 h at 110°C to optimize for recovery of phosphothreonine. The supernatant was transferred to a fresh tube and lyophilized in a Speed Vac concentrator, and washed three times with 1 ml water. The samples were electrophoresed at pH 1.9 (formic acid/glacial acetic acid/ water, 50:156:1794) on cellulose thin-layer plates using the Hunter thin layer electrophoresis unit (CBS Scientific, Del Mar, CA). The positions of the phosphopeptide standards were visualized by staining with 0.25% (wt/vol) ninhydrin in acetone.

Isolation of Triton-insoluble Cytoskeletons

Triton-insoluble cytoskeletons were isolated as described previously (10) with modifications described below. Cells (1.5×10^6) were washed twice in 15 mM Tris, pH 7.5, and resuspended in 150 μl of 100 mM MES, pH 6.8, 2.5 mM EGTA, 5 mM $MgCl_2$ and 2 mM ATP at 0°C. An equal volume of the same buffer containing 1% Triton X-100, 0.1 mg/ml PMSF, 10 mg/ml pepstatin, and 50 μg/ml TLCK was added. The suspension was vortexed 5 s, and centrifuged for 2 min in a microfuge. Supernatants and Triton-insoluble pellets were resuspended in SDS gel sample buffer, heated to 100°C for 5 min, and subjected to SDS-PAGE on 8% gels. Duplicate samples were run, with one set stained with Coomassie blue and the other set used for Western blot analysis. Coomassie blue stained myosin bands from the supernatant and pellet fractions were quantitated by densitometry. The percent myosin in the cytoskeleton was calculated for each individual sample by dividing the value for MHC in the cytoskeletal pellet by the sum of the pellet and supernatant MHC values for that sample. This "%" cytoskeleton value was then averaged for multiple-independent samples to produce the values shown in Fig. 5 *C*. The "*" for MHCK A^{++} cell line values indicate that these are estimates. MHC levels in the cytoskeletal pellets for these cell lines were consistently too low for accurate densitometry from Coomassie-stained gels.

Results

Elimination and Overexpression of MHCK A

The gene encoding MHCK A was eliminated from *Dictyostelium* by homologous recombination. The gene replacement construct contained the *Dictyostelium Thy1* gene inserted between 5′ and 3′ flanking sequences from the *mhck A* gene (Fig. 1 *A*). After introduction of the replacement construct into JH10 cells, 14 transformants were isolated for Western blot analysis with a monoclonal antibody generated against the native MHCK A. Of these cell lines, 12 had no detectable MHCK A expression.

Genomic DNA was isolated from three of the twelve antibody-negative cell lines and used for Southern blot analysis. All three cell lines displayed identical Southern blot profiles. The results for one line are shown in Fig. 1 *B*. The wild-type *mhck A* locus resides on an 8-kb XbaI restriction fragment, and in a double cross-over gene replacement, this fragment should be replaced by a 6-kb XbaI restriction fragment containing the *Thy1* gene (Fig. 1 *A*). Filters incubated with a mixed probe comprised of a 5′ ClaI–BclI fragment (probe A; see Fig. 1 *A*) and a central KpnI fragment (probe B) demonstrate the presence of the 8-kb XbaI fragment in the wild-type JH10, and the 6-kb XbaI fragment in the *mhck A* disruption cell line (Fig. 1 *B*). A duplicate filter incubated only with probe B demonstrates the presence of the 8-kb restriction fragment in JH10, but yielded no signal in the *mhck A* disruption cell line (Fig. 1 *B*), indicating that a clean gene replacement event had occurred.

MHCK A was overexpressed in the *mhck* A^- cell line by fusing the MHCK A cDNA to the actin 15 promoter in the extrachromosomal vector pLittle. Fig. 2 shows a Western blot of whole cell extracts from control (JH10 *thy*$^+$), *mhck*

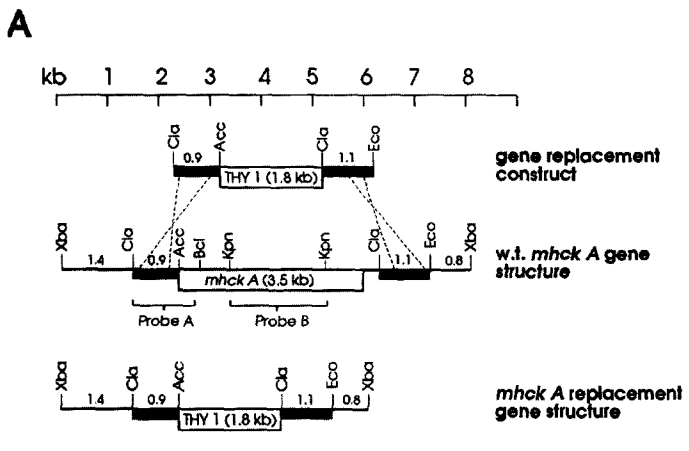

Figure 1. Generation of the *Dictyostelium mhck A*$^-$ cell lines. (*A*) Schematic of gene replacement construct, the wild-type *Dictyostelium mhck A* structure, and the disrupted *mhck A*$^-$ locus. Homologous recombination between the targeting vector and the wild-type gene eliminates the *mhck A* gene and replaces it with the *Thy1* gene. Shaded segments correspond to genomic DNA flanking the MHCK A gene which was cloned into the gene replacement construct. The dotted lines indicate predicted homologous recombination regions. (*B*) Genomic Southern blot analysis of the *mhck A* locus in parental (JH10) and *mhck A*$^-$ cells. DNA from both parental and *mhck A*$^-$ cell lines was digested with XbaI, and subjected to Southern analysis. Filters were incubated with a ClaI–BclI DNA fragment which includes 5′ noncoding DNA and some coding region sequences (Probe A) together with a KpnI–KpnI DNA fragment which lies completely within the *mhck A* coding region (Probe B), or with probe B alone.

A$^-$, and MHCK A^{++} cell lines. In the MHCK A^{++} cells MHCK A is overexpressed ~30-fold during growth phase relative to control cells as quantified by phosphorimaging of Western blots performed with ^{125}I-anti–mouse secondary antibody.

Myosin Phosphorylation

Previous studies have demonstrated that the myosin heavy chain is phosphorylated primarily on threonine and serine residues in cells lysed with Triton X-100 containing [γ-^{32}P]ATP (1, 11). We performed in vitro labeling and phosphoamino acid analysis of myosin isolated from *mhck A*$^-$ and MHCK A^{++} cell lines to determine what effect alterations in MHCK A abundance would have on phosphorylation in crude lysates. Fig. 3 shows phosphorylation of MHC in Triton X-100 cell lysates from growth phase control, *mhck A*$^-$ and MHCK A^{++} cell lines. Aliquots of growth phase cells were lysed with Triton X-100 in the presence of [γ-^{32}P]ATP, and then the myosin was immunoprecipitated as described above. Autoradiographic analysis of these samples indicates that the myosin from *mhck A*$^-$ cells was significantly less phosphorylated than myosin from control cells (Fig. 3 *B*). In contrast MHCK A^{++} cells displayed elevated MHC phosphorylation relative to the control cell line in this assay (Fig. 3 *B*). Phosphoaminoacid analysis of immunoprecipitated MHC indicated that the myosin was primarily phosphorylated on threonine residues in control cells, as well as MHCK A^{++} cells. In experiments performed with significantly higher specific activity [γ-^{32}P]ATP it was observed that the residual phosphorylation in extracts of *mhck A*$^-$ cells was also primarily on threonine (data not shown).

We also examined myosin phosphorylation during development of *Dictyostelium* cells. Control, *mhck A*$^-$, and MHCK A^{++} cells were developed on filter pads and harvested at 0, 6, and 16 h into development and phosphorylated in vitro as described above (Fig. 4). Myosin was immunoprecipitated and subjected to SDS-PAGE followed by staining with Coomassie blue. The level of MHC phosphorylation for each sample (quantified by phosphorimaging) was normalized to the amount of MHC in each sample (quantified by densitometry of the Coomassie-stained gel) so that relative MHC phosphorylation in each sample could be compared. The rate of myosin phosphorylation in crude extracts did not change significantly during development in any of the three cell lines.

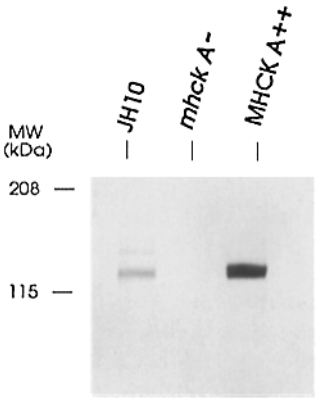

Figure 2. Expression of the MHCK A protein. Western blot analysis of MHCK A expression in control (JH10 *thy*$^+$), *mhck A*$^-$, and *mhck A*$^-$ cell line transformed with pLittle expressing MHCK A (MHCK A^{++}). Molecular size standards are shown on the left. Filter was probed with a polyclonal rabbit antiserum produced against recombinant MHCK A protein and developed enzymatically.

Myosin Assembly into the Cytoskeleton in mhck A$^-$ *and MHCK A*$^{++}$ *Cell Lines*

To address the localization properties of myosin in *mhck A*$^-$ and MHCK A^{++} cell lines Triton-insoluble cytoskeletons were isolated from the mutant cell lines. Lysed cells were fractionated by centrifugation into soluble and cytoskeletal components and subjected to SDS-PAGE.

Fig. 5 presents a typical cytoskeletal fractionation pattern for growth phase cells after SDS-PAGE and Coomassie blue staining (panel *A*) or after Western blot analysis (panel *B*). In control cells the majority of the myosin fractionated in the soluble fraction (Fig. 5, *A* and *B*, *S* lanes), while in *mhck A*$^-$ cells approximately threefold more myosin fractionated with the cytoskeleton (Fig. 5, *A* and *B*, *P* lanes). MHCK A^{++} cells had most of the myosin associated with the soluble fraction (Fig. 5, *A* and *B*, *S* lanes).

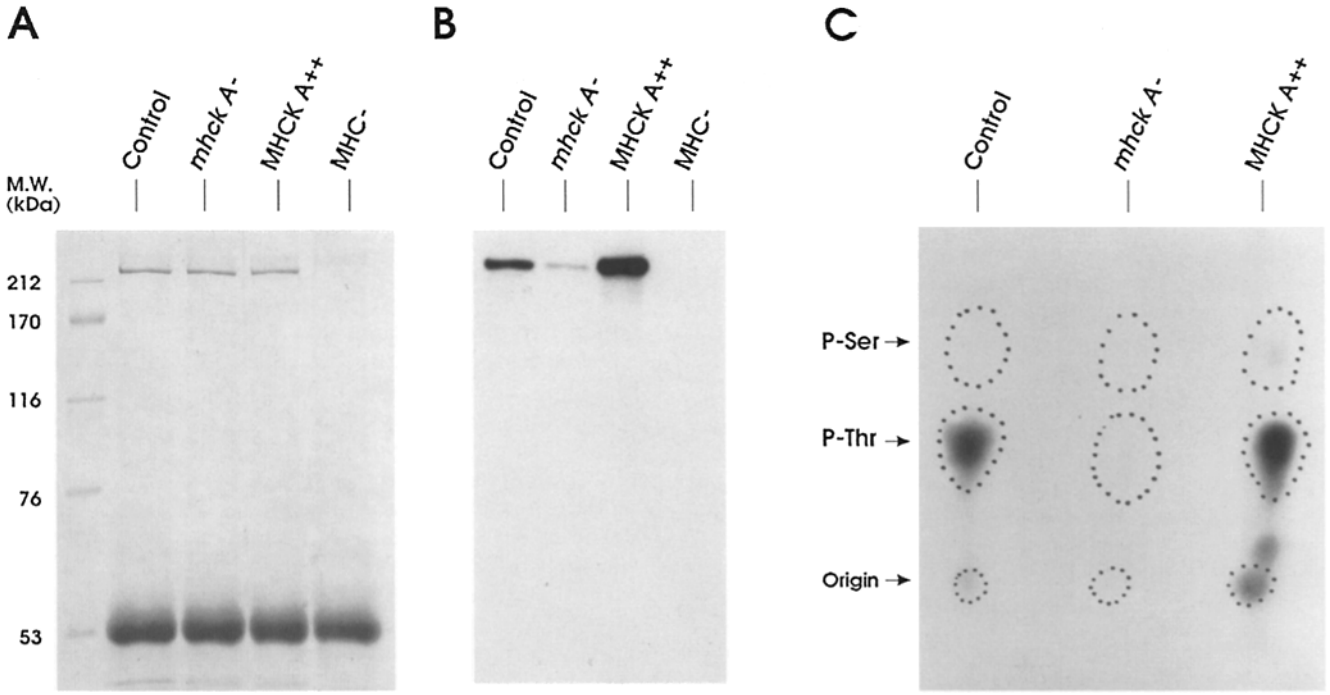

Figure 3. Phosphorylation of myosin in Triton X-100 lysates in growth phase *Dictyostelium*. After phosphorylation in Triton lysates with [γ-^{32}P]ATP, myosin was immunoprecipitated and subjected to SDS-PAGE, followed by phosphoamino acid analysis (PAA). (*A*) Coomassie-stained gel of immunoprecipitated myosin. Lane *1*, myosin from control cells (JH10 *thy*$^+$); lane *2*, myosin from *mhck A*$^-$ cells; lane *3*, myosin from MHCK A^{++} cells; lane *4*, lysates of *MHC*$^-$ cells used as negative control. The myosin heavy chain migrates at 244 kD, and the IgG heavy chain migrates at ~53 kD. (*B*) Autoradiogram of gel shown in *A*. (*C*) Phosphoamino acid analysis of myosin from control, *mhck A*$^-$ and MHCK A^{++} cell lines labeled in crude extracts. Myosin was immunoprecipitated as in *A*, and then blotted to Immobilon P and subjected to PAA analysis. Dotted lines indicate origin and positions of phosphoserine (P-Ser) and phosphothreonine (P-Thr) standards included in electrophoresis run.

Quantification of a series of Triton-fractionated samples from both growth phase cells and cells starved for 4 h was performed by densitometry of Coomassie blue–stained gels (Fig. 5 *C*). This analysis indicated that in growth phase cells 8.8% (±1.2) of the myosin from control cells fractionated with the cytoskeletal fraction, 24.9% (±2.4) of the myosin from *mhck A*$^-$ cells fractionated with the cytoskeleton, and <4% of the myosin from MHCK A^{++} cell lines fractionated with the cytoskeleton. In control cells starved 4 h 8.0% (±1.1) of the myosin was associated with the cytoskeleton, while 21.5% (±1.8) of the myosin from *mhck A*$^-$ cells fractionated with the cytoskeleton. MHCK A^{++} cells had <4% of the myosin associated with the cytoskeleton. Therefore, in both growth phase and developed *mhck A*$^-$ cells, myosin consistently overassembled into the cytoskeleton.

Growth Rates and Cytokinesis

The *mhck A*$^-$ and MHCK A^{++} cell lines grown on plastic petri dishes showed similar growth rates as compared to control cells. To assay competence for cytokinesis, cells were grown in suspension culture as previously described (10, 11). In this setting myosin function is essential for cell division (7, 24). When placed in suspension culture, cells lacking myosin II become large and multinucleated, eventually lysing. Parallel suspensions of control (JH10 *thy*$^+$) and *mhck A*$^-$ cells grew with a doubling time of 10.2 ± 0.2 h and 12.3 ± 0.2 h, respectively (Fig. 6 *A*). To directly compare growth rates of the MHCK A^{++} cell line (which carries a G418-resistance extrachromosomal plasmid) to other cell lines, control, *mhck A*$^-$, and *MHC*$^-$ cells were transfected with the vector pLittle to confer G418 resistance. Analysis of doubling times was then performed with all cell lines in the presence of G418 (8 μg/ml) to adjust for the slower growth that occurs in the presence of this antibiotic (Fig. 6 *B*). Control-pLittle cells doubled every 20.8 ± 0.5 h while *mhck A*$^-$-pLittle cells showed a small but reproducible decrease in growth rate in late log phase and doubled every 23.6 ± 0.7 h. *mhck A*$^-$ -pLittle cells remained of normal size, were generally mononucleated, and eventually grew to a similar density as control cells. MHCK A^{++} cells displayed a slower than normal growth rate doubling every 27.9 ± 2.6 h, displayed frequent multinucleation while in suspension culture, and grew to a lower final density as compared to control-pLittle and *mhck A*$^-$-pLittle cells. MHCK A^{++} cells increased in size during the first several days in suspension and with some cell lysis. However, in contrast to myosin null cell these cells are able to grow in suspension indicating that these cells are at least partially competent to carry out cytokinesis.

Development

Myosin null cells arrest at the mound stage and are unable to complete development, indicating that myosin function is essential for morphogenesis (7). To assess roles of MHCK A during development, control, *mhck A*$^-$, and

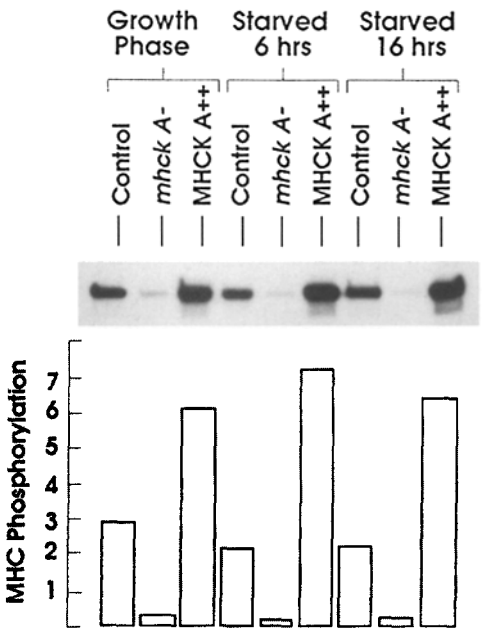

Figure 4. Phosphorylation of myosin in growth phase and starved *Dictyostelium*. Control cell lines (JH10 *thy*$^+$), *mhck* A^-, and MHCK A^{++} were developed on filter pads for 0, 6, or 16 h. Cells were collected and lysates were phosphorylated in vitro, followed by immunoprecipitation of myosin and SDS-PAGE. The top portion of figure shows autoradiogram of immunoprecipitated myosin from each time point. Bar graph indicates the relative myosin heavy chain phosphorylation level of each sample as determined by phosphorimaging and densitometry (^{32}P counts/U myosin).

MHCK A^{++}cell lines were allowed to develop on filters. The *mhck* A^- cells were able to develop normally and formed fruiting bodies at rates (~24 h) comparable to control cells (Fig. 7, *A* and *B*). In addition, *mhck* A^- cells displayed slug migration rates similar to control cells when tested on water-agar plates during phototaxis (35) (data not shown). MHCK A^{++} cells arrested at the mound stage and were unable to complete development (Fig. 7 *C*). In some experiments partial development was observed in MHCK A^{++} cells after 4–5 d on filter pads, but this pattern was not consistent.

Discussion

We have used targeted homologous recombination of the *mhck A* gene to produce *Dictyostelium* cells that lack the MHCK A protein. Southern blot analysis of these cells indicated that the *mhck A* gene was eliminated and a single copy of the *Thy1* gene was inserted at the *mhck A* locus. Western blot analysis of the mutant cell lines showed that no MHCK A polypeptide was expressed in *mhck* A^- cells, and revealed a 30-fold increase in MHCK A expression in *mhck* A^- cells transfected with the MHCK A gene on an extrachromosomal vector.

The MHCK A protein kinase is unusual in that at the primary sequence level it displays no detectable similarity to known classes of protein kinases (13). The results presented here provide strong evidence that the biochemically identified MHCK A protein does function as an MHC kinase in *Dictyostelium*, and that it has a specific role regulating myosin localization in vivo.

In previous studies MHC phosphorylation analysis was performed in crude lysates, or in lysates partitioned into cytoskeletal and soluble fractions (1, 2). Those studies demonstrated that virtually all MHC kinase activity in crude lysates cofractionates with the Triton-insoluble cytoskeleton. Furthermore, it was observed in those studies that when lysates were prepared from cells stimulated with the chemoattractant cAMP, a transient increase in myosin assembly into the cytoskeleton occurred ~40 s after stimulation. This increase in assembled myosin was observed to coincide temporally with an increase in MHC phosphorylation rate in such lysates. It was proposed that myosin assembly into the cytoskeleton in response to cAMP might increase substrate availability to cytoskeletally associated MHC kinase activities, resulting in the observed increase in MHC phosphorylation rate (1).

The in vitro phosphorylation analysis presented in the current work indicates that the majority of the in vitro MHC kinase activity in Triton X-100 lysates is due to MHCK A. In *mhck* A^- cells, ~90% of the MHC kinase activity in such lysates is absent. This result, together with the elevated level of MHC kinase activity in MHCK A^{++}cell lysates, indicates that MHCK A is the major activity that phosphorylates MHC in crude lysates. It is important to note the same pattern was observed in lysates of growth phase cells, and in lysates of cells developed on filter pads for 6 or 16 h. MHCK A appears to be the major MHC kinase activity in crude lysates in all these settings, and appears to be the major activity previously observed in in vitro studies (1). We believe that in vitro phosphorylation in this assay represents phosphate incorporated into myosin which was not phosphorylated at the time of cell lysis. If MHCK A^{++}cells have elevated levels of in vivo MHC phosphorylation, there may be less available substrate in these extracts, so that the increase in phosphorylation is smaller than one might expect given the 30-fold overexpression of MHCK A in these cells. The converse also may be true in the *mhck* A^-cells; reduced MHC phosphorylation at MHCK A target sites may allow an increase in the apparent phosphorylation rate in vitro by other kinases present in the *mhck* A^- cells. Cold ATP chase experiments (unpublished observation and see reference 1) indicated very little dephosphorylation of MHC in these Triton extracts, suggesting that no significant turnover of phosphate occurs in these assays.

These results do not preclude roles for other MHC kinase activities in the in vivo control of myosin function. Strong in vitro evidence exists for the importance of the 84-kD PKC-like MHCK in controlling myosin function (28, 29), and it is likely that the developmentally expressed PKC-like enzyme has developmental-specific roles as well. The predominant role of MHCK A in MHC phosphorylation in crude extracts may be due to either high activity of this enzyme in such lysates, or to a physical interaction between MHCK A and myosin which enhances the relative activity of this kinase in vitro.

The Triton-resistant cytoskeleton analysis of the *mhck* A^- and MHCK A^{++} cell lines provide further evidence that this kinase plays a key role in controlling myosin assembly into the cytoskeleton. Previous analyses of site-

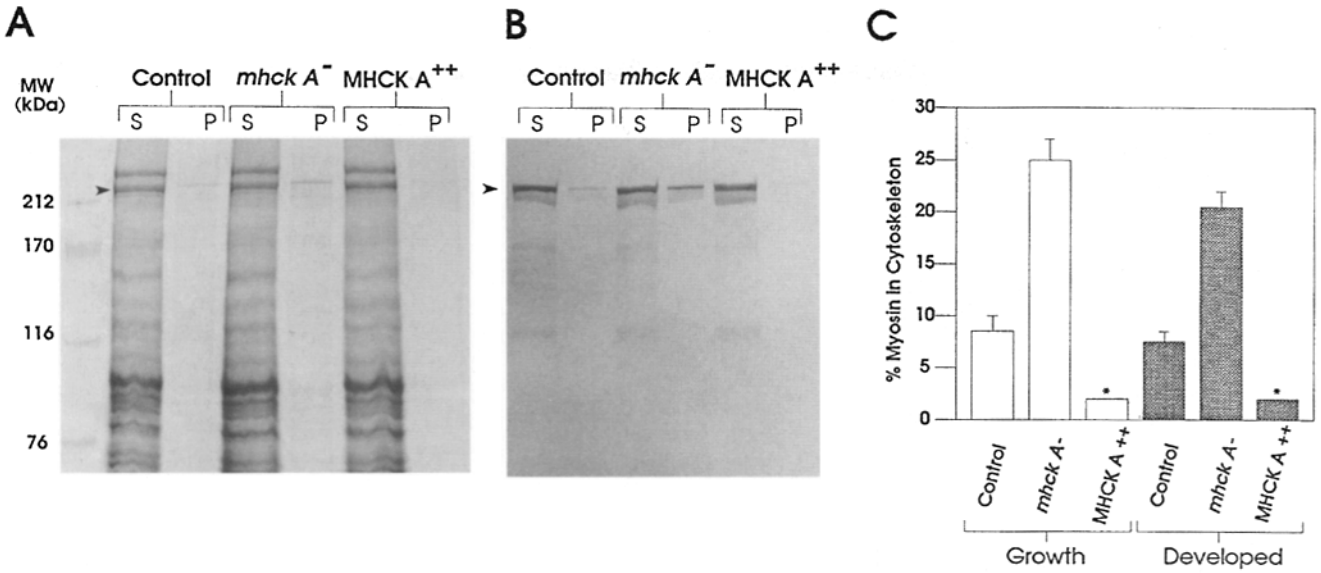

Figure 5. Isolation of Triton-insoluble cytoskeletons. Cells were lysed with buffer containing Triton X-100 at 0°C and centrifuged at 4°C for 2 min to pellet cytoskeletal ghosts. The soluble cytosolic fractions (S) and the cytoskeletal pellet (P) were subjected to SDS-PAGE and either stained with Coomassie blue (*A*), or transferred to nitrocellulose filters for Western blot analysis probed with polyclonal anti-myosin antiserum (*B*). Arrowheads indicate position of MHC. Samples in panels *A* and *B* are from growth phase cells. Panel *C* presents densitometry values for cytoskeletal fractions from growth phase and 4 h developed cells. Error bars represent SEM, $n = 12$. Asterisks indicate values for which the MHC bands were too faint for accurate densitometry.

directed mutants of MHC phosphorylation sites provide a useful model for assessing the phenotypes of *mhck A*$^-$ and MHCK A^{++} cell lines. Cells containing myosin which is nonphosphorylatable at MHCK A target sites (3X ALA cells) or which is pseudophosphorylated by introduction of aspartate residues (3X ASP cells) display overassembly and underassembly of myosin into the cytoskeleton, respectively (11). In this context the overassembly of MHC into the cytoskeleton in *mhck A*$^-$ cells indicates that MHCK A functions during both growth and development to modulate myosin assembly into the cytoskeleton. This interpretation is further supported by the underassembly of myosin in MHCK A^{++} cells. It should be noted that absolute myosin recovery in Triton-cytoskeletal fractions varies depending on exact conditions (ATP concentration, Ca^{2+} concentration, temperature, etc.; unpublished observations and see references 8, 11, 33). Of more importance is the relative difference between different cell lines assayed in parallel under identical conditions. In this regard, clear differences are present between the wild-type, *mhck A*$^-$, and MHCK A^{++} cells.

Analysis of growth rates in suspension revealed a slight but reproducible decrease in rate in *mhck A*$^-$ cells. Although small, this effect is consistent with a modulatory role for MHCK A in either cytokinesis or growth in general. It is noteworthy that a similar but more severe effect was observed previously in the 3X ALA MHC mutants, which display more dramatic myosin overassembly into the cytoskeleton (11). Cells expressing the 3X ALA MHC had ~80% of cellular myosin associated with the Triton-

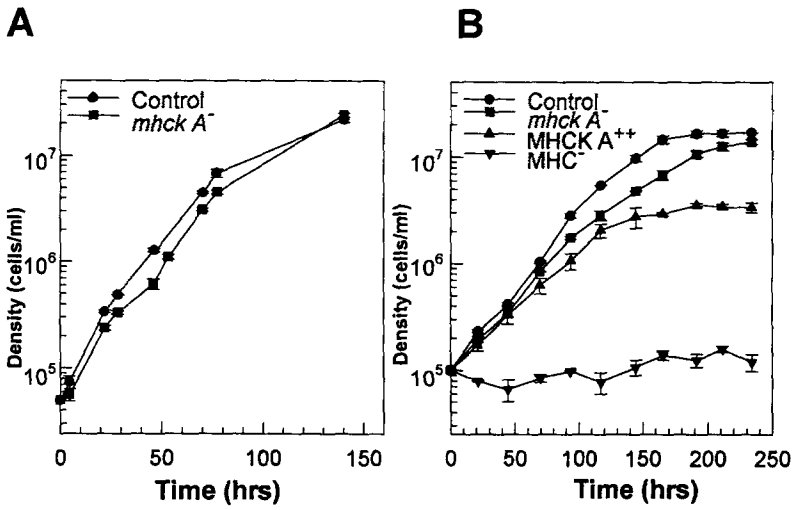

Figure 6. Growth curves of cells in suspension cultures. (*A*) Cultures inoculated at 0.5×10^5 cells/ml were grown in suspension in HL5 and rotated at 200 rpm. (*B*) Cultures inoculated at 10^5 cells/ml were grown in suspension in HL5 with 8 μg/ml G418 (all cell lines transfected with pLittle or pLittle derivatives) and rotated at 200 rpm. Density was determined by hemocytometer counts. Error bars represent SEM for triplicate samples. Error is smaller than symbols for some samples.

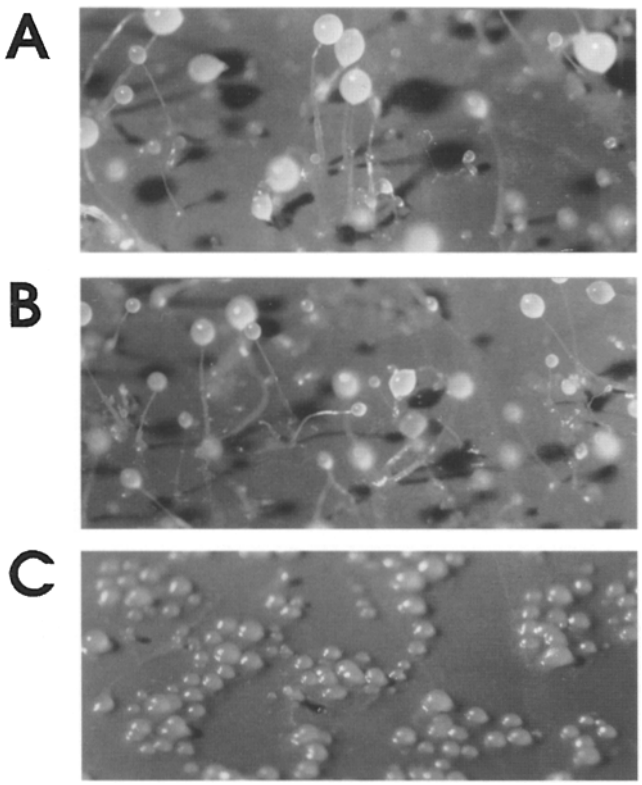

Figure 7. Synchronous morphogenesis on filters. Synchronous morphogenesis was performed by plating cells on filter pads in Lower Pad Solution (35). (*A*) Control (JH10 *thy*$^+$), (*B*) *mhck* A^-, and (*C*) MHCK A^{++}. Development appeared complete by 24 h, photograph was taken at 48 h.

insoluble cytoskeleton, and displayed very long doubling times in suspension relative to control cells.

Overexpression of MHCK A produces a partial defect in cytokinesis, again similar but less severe than that of the previously characterized 3X ASP MHC mutants which express pseudophosphorylated MHC (11). No detectable defect was observed in rates of development in *mhck* A^- cells, while MHCK A^{++} cells were blocked at the mound stage. All of the growth and developmental phenotypes observed in the current study are consistent with the phenotypes previously observed in 3X ALA and 3X ASP myosin cells, and are furthermore consistent with the model that MHCK A participates in regulation of myosin localization in vivo. These results also indicate that partial overassembly of myosin into the cytoskeleton is not highly deleterious, but that hyperphosphorylation of MHC by MHCK A (in MHCK A^{++} cells) inhibits assembly into the cytoskeleton and that this significantly reduces myosin function in vivo.

The relationship between MHCK A and other MHCKs, particularly the 84-kD PKC-like MHCK, is not well understood. In addition to that MHCK, other activities have been observed that phosphorylate MHC on serine as well as threonine (20, 25). A firm understanding of the relationships between these kinases will require cloning and gene disruption of the genes encoding these activities. A notable discrepancy between the properties of 3X ALA cells and *mhck* A^- cells is that the former cells assemble ~80% of their myosin into Triton-resistant cytoskeletons while the latter cells assemble only ~25%. While there is clear overassembly of myosin in the *mhck* A^- cells, this difference implies that there are other physiologically important MHC kinases which participate in regulation of myosin localization during both growth and developmental stages. These results also imply that the other activity or activities phosphorylate the same target sites that were originally mapped in vitro for MHCK A (residues 1823, 1833, and 2029), as removal of these target sites eliminates control of localization in vivo (e.g., 3X ALA myosin).

Overall these results indicate a role for MHCK A in regulating myosin localization during both growth and development, but also indicate a role for other kinases. Further analysis of the relative roles of the 84-kD PKC-like MHCK vs other partially characterized activities will help establish the general mechanism by which *Dictyostelium* cells regulate myosin function and localization.

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